A STUDY ON INFECTIONS IN HAEMATOLOGICAL MALIGNANCIES AND DISORDERS ASSOCIATED WITH NEUTROPENIA

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CERTIFICATE

Certified that the dissertation entitled **"A STUDY ON INFECTIONS IN HAEMATOLOGICAL MALIGNANCIES AND DISORDERS ASSOCIATED WITH NEUTROPENIA"** is a bonafide work done by **Dr. K. USHA KRISHNAN,** Postgraduate, Institute of Microbiology, Madras Medical College, Chennai, under my guidance and supervision in partial fulfillment of the regulation of the Tamil Nadu Dr. M.G.R Medical University for the award of **M.D. Degree, Branch – IV (Microbiology)** during the academic period of May 2005 to March 2008.

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CONTENTS

Introduction

INTRODUCTION

Many of our body surfaces are colonized with a wide variety of microorganisms without apparent detriment. An infection involves the growth and multiplication of microorganisms that result in damage to the host.

The first line defenses are the external and internal body surfaces that are relatively in direct contact with the external environment.

Traditionally, the inner defenses have been grouped into two, more or less distinct functional systems. Innate immunity refers to any inborn resistance that is already present the first time a pathogen is encountered.

Acquired immunity refers to resistance that is weak on first exposure, but that increases dramatically with subsequent exposure to the same specific pathogen.

The two arms of the immune system are humoral and cell mediated immunity.

The immune system provides the human host with the ability to mount a specific protective response to the presence of a microorganism.

Patients with haematological malignancies comprise a special group in whom all the aspects of immunity are compromised as a result of the underlying malignancy or due to the therapeutic interventions employed to manage it.

In many cases there are multiple factors that predispose these patients to infections, such as neutropenia induced by therapy or bone marrow involvement, hypogammaglobulinemia, T-cell dysfunction and mucosal damage. In addition newer therapy has changed the spectrum of infection that is seen in these patients.[1]

Neutropenia resulting from cytotoxic chemotherapy is the most common risk factor for severe bacterial infection in haematological malignancies.[2]

Similarly common procedures such as venepunctures, bone marrow aspiration and insertion of central venous access devices, disrupt the integument and provide nidus for colonization.[3]

The degree of neutropenia is directly related to the incidence of serious bacterial and fungal infection.[4]

There is a significant increase in the incidence of serious infection once absolute neutrophil count (ANC) falls, below 1500 cells / mm^3.

The duration of neutropenia also contributes significantly to the risk of serious infection. The risk is significantly greater at lower neutrophil counts. 100% patients with ANC<100 cells/mm^3 lasting 3 weeks or more develop documented infection.

Some malignancies are associated with specific immune defects that predispose to infections with particular pathogens.

Patients with acute leukemia have increased risk of severe gram negative bacterial infections as a result of quantitative or functional neutropenia.

Patients with chronic lymphocytic leukemia and multiple myeloma are susceptible to invasive bacterial infections from *Staphylococci* and *Streptococci.* Conversely patients with lymphoma have abnormalities of the cellular immune system resulting in an increased risk of viral infection and fungal infections.[4]

Infections occur in 30-50% of these patients, most often in patients who have advanced disease and it remains a major problem in the management.[5]

The overall incidence of infection in patients who have chemotherapy is 0.25-0.5 episodes per patient per year and increased upto 1.8 episodes / year in patients who have advanced disease.[6]

In patients who have acute leukemia, infection alone accounts for 70% of deaths, haemorrhage and infection together for another 10%.[7]

Neutropenic infections are an emergency and the patients should be started on empirical antibiotic therapy without delay immediately after appropriate cultures are taken. Close monitoring of the patients is however essential and modifications in therapy must be made according to the clinical and microbiological findings.

The majority of the treatment failures, relapses and super infections are generally attributed to resistant infective organisms such as Methicillin resistant *Staphylococcus* and *Pseudomonas sp.* or disseminated fungal infections.[8]

Hence a study was undertaken to identify the organisms causing infections in Haematological malignancies and disorders, and their antibiotic susceptibility patterns and also to monitor the emergence of resistant strain. This will help to modify the empirical antibiotic regimens if necessary.

Review of literature

REVIEW OF LITERATURE

Patients with haematological malignancies and disorder comprise nearly 3% of all hospital admission.

Infection is a major cause of morbidity in patients with acute leukemia due to therapy induced neutropenia.[9]

Haematological conditions predispensing to infection

Leukemia are malignant neoplasm of the haematopoitic stem cells, characterized by diffuse displacement of the bone marrow by neoplastic cells.

Acute leukemias are characterized by accumulation of leukemic blasts resulting from clonal expansion of transformed stem cells as well as a failure of maturation into functional end cells.

ALL is primary a disease of children and young adults. It consists of 80% of childhood acute leukemia.[10]

AML constitute only 20% of childhood leukemia. 60% of patients with AML achieve complete remission with chemotherapy but only 15-30% of these remain free from disease only for 5 years. Therefore on a increasing number of patients are being treated with Allogenic Bone marrow transplantation.[10]

CML accounts for 15-20% of all cases of leukemia,[10] a disease primarily of adults between ages 25-60 years with peak in fourth to fifth decade.

Lymphomas are malignant neoplasm characterised by proliferation of cells native to the lymphoid tissue that is lymphocytes, Histiocytes and their precursor and derivatives.

Hodgkins lymphoma is distinctive by the presence of the reed sternberg giant cells in addition to the presence of non-neoplastic inflammatory cells[11].

Non Hodgkins lymphoma – In the majority of them T cells reveal a characteristic chromosomal translocation.[11]

Approximately 50% of lymphomas are associated with a history of immunological disorder.

Haematological disorder includes disorders due to bone marrow dysfunction or disease of various cells in the blood like RBC, WBC, platelets leading to their lowered counts or their dysfunction.

In bone marrow failure pancytopenia results from deficient haematopoises as distinguished from blood count depression due to peripheral destruction of red cells, platelets and granulocytes.

Factors predisposing to infection in Haematological condition

Common host defense impairement in Acute leukemia are neutropenia / neutrophil dysfunction altered mucosal and skin integrity thrombocytopenia (causing poor wound healing) altered cellular and humoral immunity (treatment related)[12]

During antineoplastic treatment, cytotoxic agents are frequently administered in combination with other immunosuppressive therapies, such as corticosteroids and radiation therapy. Several of the cytotoxic agents notably methotrexate, cyclophosphamide, 6-mercaptopurine, and azathioprine, impair cell-mediated immunity.[12] Many of the drugs themselves can impair other immune parameters, including humoral responses and can produce quantitative phagocyte defects.

Exogenous administration of glucocorticoids leads to increased susceptibility to infection. The degree of immunosuppression depend on the dose and duration of use. The major effect of steroids on granulocyte function is a decrease in chemotactic activity.

Radiation therapy has been associated with granulocyte dysfunction and delayed wound healing.[12]

Interleukin-2 administration depresses host defense, by decreased production of superoxide, decreased chemotaxis, decreased FC receptor γ-III expression of granulocytes[12]

In early B cell CLL, the infection risk is mainly related to unbalanced immunoglobulin chain synthesis and resultant hypogammaglobulinemia.

In patients having advanced CLL, with purine analog and monoclonal agent (rituxumab or alemtuzumab) therapy, neutropenia and defects in CMI are additional factors predisposing to infection.[13]

Hodgkins disease, followed by NHL is the most commonly encountered malignant disorder associated with impaired CMI, The extent of impairment is compounded by the administration of immunosuppressive therapy.[12]

In multiple myeloma, Hypogammaglobulinemia and other factors associated with aggressive therapy are responsible for defects in Humoral immunity.[5] Functional neutropenia occur in myelodysplastic syndrome. In Aplastic anaemia, bone marrow failure leads to pancytopenia. Neutropenia and abnormal cell mediated immunity resulting from therapies (eg. steroid anti thymocyte globulin, cyclosporine, bone marrow transplantation) are all risk factors, predisposing to infection.[12]

ORGANISMS LIKELY TO CAUSE INFECTION IN GRANULOCYTOPENIC PATIENTS[5]

Gram Positive Cocci	Gram Positive Bacilli	Gram Negative Bacilli
S. epidermidis	*Diphtheriods*	*E.coli*
S. aureus	*C. jeikeium*	*Klebsiella sp*
S. viridans		*Pseudomonas aeruginosa*
Ent. faecalis		*Pseudomonas sp.*
Str. pneumoniae		*Enterobacter sp.*
		Serratia sp.
		Acinetobacter Sp.
		Citrobacter sp.

Fungi

Candida sp.

Aspergillus sp.

Fever is the principal sign of infection in neutropenic patient and frequently may be the only evidence of infection.[3,2]

Neutropenia

Neutropenia is defined as a neutrophil count <500 cells / mm^3 or < 1000 cells / mm^3 with expected decrease to < 500 cells / mm^3 within 48 hours.[2] Studies have shown that there is an inverse relationship between the number of circulating neutrophils and the incidence of infection. As the neutrophil count decreases to < 1000 cells / mm^3, the incidence of infections increases markedly. The risk of severe infectious complications such as blood stream infection is greatest when the neutrophil count drops below 100 cells / mm^3.[13]

The duration of neutropenia is also a major determinant of the risk of infection. Profound and prolonged neutropenia (ie<500 cells / mm^3 for more than 10 days) is considered to be a major risk factor for both primary and secondary bacterial or fungal infections.[13]

Thus there are no immunodeficiencies associated to each disease, instead several immunitarian deficiencies can be associated in a single clinical entity.[14]

Febrile episodes during neutropenia are defined as an oral temperature of 38.3°C (100F) in the absence of other non-infectious cause of fever such as administration of blood / blood products) pyrogenic drugs, cytotoxic therapy (Amphotericin B) the underlying disease, thromboembolic, thrombophlebotic or haemorragic events.[15]

The pattern of fever in the presence of neutropenia is nonspecific and non pathognomonic of any specific type of infections.

Classically infection have been subdivided into three main categories.[6]

I. Microbiologically documented infection (MDI)

Most episodes of MDI consists of bloodstream infections and may be either primary or secondary to a proven focus of infection (eg pneumonia, cellulitis, catheter related infection, UTI) and it accounts for 25-35%[16,17]

II. Clinically documented infections (CDI)

Defined by the presence of a site of infection (eg. pneumonia, cellulitis, oropharyngeal mucositis, enterocolitis, catheter site infection) without microbiological proof of the nature of infection and it accounts for 20-30%.[16,17]

III. Fever of unknown origin (FUO)

Defined as a febile episode that is not accompanied by clinical or microbiological evidence of infection and it accounts for 40-60%.[16,17]

Neutropenic patients may have unusual presentations of infection because of their inability to mount an adequate inflammatory response

and their susceptibility to infection caused by less virulent organism. If not treated promptly, infection in neutropenic patients can progress rapidly.[18]

Site of infection

The most frequent sites of infection in neutropenic cancer patients with haematological malignancies by decreasing order of frequency, the blood stream, the oralcavity and nasopharynx, the skin, soft tissue, the respiratory tract, the gastrointestinal tract, and the urinary tract.[6]

Unusual sites of infection include, typhilitis, perirectal infections and atypical forms of cellulitis.[18]

Blood Stream Infections

Nosocomial blood stream infection in neutropenic patients is significantly associated with an excess length of hospital stay, extra costs, and excess mortality. Severe sepsis and septic shock are closely correlated with adverse outcome.[14]

Bacteria are the most frequent blood, isolates, accounting for over 90%. Gram positive isolates includes *CONS, Viridans Streptococci, S.aureus.* Among gram negatives *E.coli, Klebsiella spp.* and other *enterobacteriaceae* are predominant.[19]

Body sites often associated with infection in the neutropenic patients are those associated with integumental surface (skin, upper / lower respiratory tract, upper & lower gastrointestinal tract).

Skin and Soft tissue Infections

Cellulitis and acute inflammation of the skin are most often caused by infection with group A *Streptococci / S. aureus*.[4]

Although cellulites may be circumscribed in normal hosts it can spread in neutropenic patients. Rapid progression of local infection with blood stream invasion and septicemia is frequent.[4]

Patient who are neutropenic and have received antibiotics for other reasons may develop cellulitis due to unusual organisms (*E.coli, Pseudomonas, fungi*).[4]

Stomatitis of the oral cavity is a major complication of cancer chemotherapy, signs being erythema, edema followed by secondary infection further complicating management.[4]

Patients undergoing remission induction for AML or bone marrow transplantation with a history of Herpetic stomatitis or who are IgG seropositive for Herpes simplex virus are at risk for severe herpetic mucositis.[20]

Skin specific syndromes and ecthyma gangrenosum located in non pressure areas is often associated with *Pseu. aeruginosa* bacteremia, but may be caused by other bacteria also.[4]

Enteric infections

Factors favouring enteric infections are mucositis from cancer treatments, reduced stomach acidity, reduced intestinal motility, reduced mucosal, humoral and cellular immunity.[20]

Ingested microorganism can cause systemic infections in these patients even in numbers smaller than the usual infective dose.[20]

Invasive enteric bacterial infection of the gut due to *Salmonella* or *Shigella* species are relatively uncommon in neutropenic patients.[20]

Two clinical entities to be considered in febrile neutropeunic patients with abdominal pain and diarrhoea are toxigenic enterocolitis due to toxin elaborated from an overgrowth of clostridium difficile and neutropenic enterocolitis (Typhilitis).[20]

Bacteremia with enteric microorganisms (*E.coli, Klebsiella* species *Pseu. aeruginosa*) is associated with Typhilitis in upto 28% of cases.

Respiratory Tract Infections

Damage to local and systemic host defenses of the lung makes the immunocompromised patient vulnerable to inhaled microorganisms. Neutropenia is associated with gram negative bacilli pneumonia. Prolonged neutropenia increases the risk of invasive aspergillosis and other unusual mycotic agents.

Cellular immunodeficiency is associated with intracellular microorganisms including *Mycobacteria sp. Nocardia sp. Legionella sp, Cytomegalovirus.* Humoral immunodeficiency predisposes to *Hemophilus influenzae* and *S. pneumoniae* infection.[21]

Fungal infections are increasing in frequency, among patients with haematological malignancies. The fungi which cause most of the infections in cancer patients are *Candida spp.* and *Aspergillus spp.*

Many factors predispose patients to fungal infections, including neutropenia, lymphopenia, gastrointestinal ulceration, interavenous catheters and steroid therapy. *Candida spp.* cause 5 major types of infection, dermatitis, thrush, gastro-intestinal, primary organ and disseminated infection. *Aspergillus spp.* and phycomycetes cause pulmonary, disseminated or rhino-cerebral infection. *Cryptococcus neoformans* usually cause meningitis, but may cause pneumonia or disseminated infection.[22]

Enhancement of host defenses with growth factors and cytokines may decrease the incidence and improve the final outcome of respiratory infections in the immunocompromised host.

Successful treatment depends on the type of pathogen, status of host defense and early appropriate use of ideal antibiotic. Therefore, selection of potent, broad spectrum agents when iniating empirical antimicrobial therapy in the neutropenic patient is critical.[23,24] Hence a study of common organisms causing infections in haematological malignancy patients and their antibiotic sensitivity pattern is essential for effective treatment.

Aims and objectives

AIMS AND OBJECTIVES

- To isolate and identify the bacterial and fungal agents causing febrile episodes in patients with neutropenia following chemotherapy for Haematological malignancies.
- To identify bacterial and fungal pathogens in patients with other haematological disorders
- To study the antimicrobial susceptibility pattern of isolates
- To evaluate an ideal protocol for standard antibiotic therapy in neutropenic patients
- To analyse the correlation between neutrophil counts, etiological agents and episodes of infection among different age groups
- To identify factors influencing mortality in these patients

Materials and methods

MATERIALS AND METHODS

Period of Study

This prospective study carried out over a period of 1 year from April 2006 to May 2007.

Place of study

This study was undertaken at the Institute of Microbiology, Madras Medical College, Chennai and The Department of Haematology Government General Hospital, Chennai.

Study Group

A total of 94 patients both male and female were included in the study comprising of

Acute Myeloid Leukemia	47
Acute Lymphatic Leukemia	21
Chronic Myeloid Leukemia	4
Chronic Lymphocytic Leukemia	1
Lymphoma	6
Haematological disorders	15

Inclusion criteria

All patients having episodes of fever 38.4°C and above and having any systemic complaints and / or local sepsis and belonging to the following category.

1. Patient having hematological malignancies on chemotherapy
2. Patient with neutropenia due to hematological disorders

Neutropenia is frequently classified as mild, moderate or severe.

Mild neutropenia corresponds to an absolute neutrophil count of 1000-2000 x 10^9/mm.^{3} Moderate neutropenia is defined as an absolute neutrophil count of 500-1000x10^9/mm^3 and in severe neutropenia the absolute neutrophil count is <500 x 10^9/mm^3.[20]

Methodology

i. Collection of specimen

ii. Macroscopic examination

iii. Microscopic examination

iv. Culture procedure

v. Identification of organisms

vi. Antibiogram

vii. Tests for detection of β-lactamase producing organisms

Collection of Specimens

Blood

After choosing the vein to be drawn, the skin over the venepuncture site is disinfected first with 70% alcohol in a circular motion. Allowed to air dry. Then 2% tincture iodine (or povidone iodine) is applied and allowed the iodine to dry on the skin for 1 minute.[25]

After disinfecting the venepuncture site, about 5 ml of venous blood was drawn from three different sites using sterile disposable needle and syringe and were innoculated onto 50 ml of BHI broth. (Appendix 1)

Based upon the clinical manifestation, serum was collected for testing antibodies to HIV and HBsAg (Appendix 2) and for serological test for enteric fever, brucellosis and leptospirosis. (Appendix 3)

Peripheral Blood Smear

Thick and thin smears were made from the peripheral blood, to look for the presence of malarial parasite.

Thin film-One drop of blood was placed over the glass slide, with the help of spreader at 45° angle, thin smear was made.

Thick film-One drop of blood was placed on the same slide on the other corner. By using corner of the spreader circular thick smear was made.

Sputum

Patients were instructed to have mouthwash and gargle with sterile distilled water and to cough when the sputum was felt in the throat, and to spit the material directly into a wide mouthed sterile container.

Tongue ulcer scrapings

Material from lesion was collected with sterile swab.

Pleural Fluid

Collected by aspiration with a needle and syringe under aseptic precaution.

Wound swab

Using sterile swab the specimens were collected and transported to the laboratory without any delay.

Faeces

The specimens were collected in a sterile wide mouthed bottle.

ii. Macroscopic Examination

Sputum

Sputum was examined for colour and consistency and presence of mucus, mucopurulant and frothy material.

Pleural fluid

Aspirated pleural fluid was examined for consistency, colour, turbidity and presence of blood.

Faeces

Faeces was observed for the presence of mucus, pus, blood and for the segments of tapeworm.

iii. Microscopic Examination

Blood

Both thick and thin smears were stained by leishman's method (Appendix 4) and examined for malarial parasites.

Gram stain

Smears from sputum, wound swab, tongue scrapings and pleural aspirates were all stained by Gram's method and examined for the presence of squamous epithelial cells, polymorphonuclear leucocytes,

mononuclear leucocytes, gram positive, gram negative bacteria and yeast cells.

Ziehl Neelson Stain

Sputum specimens were subjected to concentration technique by Petroff's method.

Petroff's method

Sputum is incubated with an equal volume of 4% sodium hydroxide solution at 37°C with frequent staking till it becomes clear, on an average for 20 minutes. It is then centrifuged at 3000 rpm for 20 minutes and the sediments neutralised with N/10 Hcl and used for preparation of smear.[26]

The smear stained by Ziehl Neelson method and examined for acid fast bacilli.

Faeces

Saline and Iodine mount preparation of faeces were done and observed under low and high power objectives for the presence of trophozoites, ova and cyst.

iv. Culture Procedure

5 ml of blood from 2 sites were inoculated into 50 ml of Brain Heart infusion broth. From the third site 3 ml was inoculated into thioglycolate broth (Appendix 1) and 2 ml was used for obtaining serum by transfering onto a sterile tube.

The blood culture bottles were incubated at 37°C. Subcultures were made at 24 hrs, 48 hrs and after 5 days and on 7^{th} day onto MacConkey, Blood agar and Nutrient agar plate (Appendix 5) incubated aerobically. Blood agar plate was incubated in 10% Co_2 in candle jar.

Subcultures from thioglycollate broth were made on neomycin Blood agar plates (Appendix 5) and one set incubated aerobically and another set incubated anaerobically in McIntosh jar. Aerobic plates were examined at 24 hrs and 48 hrs and anaerobic plates at 72° hrs, for growth.

Sputum

Sputum was plated onto MacConkey, Chocolate agar plates. (Appendix 5) Incubated at 37°C for 24 hrs. CAP was incubated in 10% CO_2 in candle jar. Plates were examined after 24 hrs for the presence of growth. If growth was present isolates were identified based on colony morphology, gram stain and standard biochemical reactions. (Appendix 6)

Sabouraud's Dextrose Agar

Each sputum specimen was inoculated onto 2 sets of SDA slopes, (Appendix 7) one set with 50 mg / 100 ml of cycloheximide (Actidione) and 16 μg/ml of chloramphenicol and the other set without cycloheximide and chloramphenicol. One tube from each set was incubated at 37°C and the other tube incubated at 25°C.

The culture tubes were examined for growth daily for a week, and twice a week for further four weeks before discarding it as sterile.

If growth was present, the fungal isolates were further identified by gram stain and lactophenol cotton blue mount.

Tongue Scrapings

Were inoculated onto 2 SDA slopes without cycloheximide and antibiotic and one tube incubated at 25°C and the other at 37°C. Examined after 24 hrs for growth. If growth was seen, it was confirmed by Gram stain and LPCB. Further candida speciation was done by germ tube test and sugar assimilation test.

Pleural fluid was also inoculated onto MacConkey, Chocolate agar plates and incubated at 37°C for 24 hrs and observed for growth.

Wound swabs were inoculated onto MacConkey and Chocolate agar plates and thioglycolate broth.

Subcultures were made from thioglycolate broth, onto 2 sets of Neomycin Blood agar plates and one set incubated at 37°C aerobically and another set incubated anaerobically in McIntosh jar. Plates were examined and processed as per routine procedure.

Faeces was inoculated onto MacConkey and XLD (Appendix 5) plates, both directly from specimen and after enrichment in Selenite F broth. (Appendix 1)

After 24 hrs isolates were identified by conventional Biochemical tests and antibiotic susceptibility test was put up as required.

Detection of Antimicrobial Susceptibility Pattern

Antimicrobial susceptibility test was done on Mueller – Hinton agar (Appendix 5) by Kirby - Bauer disk diffusion method as recommended by NCCLS.

Test inoculum 0.5 Mc Farland Lawn culture

Incubation – 37°C for 16-18 hrs

Control Strains:

Staph aureus – ATCC 25923

E.coli ATCC – 25922

Pseudomonas aeruginosa ATCC 27853

The antibiotics used

Antimicrobial Agent	Inhibition zone in mm		
	Resistant	**Intermediate**	**Sensitive**
Penicillin G (IOU)	< 20	21-29	> 30
Ampicillin (10 mg)	< 20	21-29	> 30
Oxacillin (1 mg)			> 13
Gentamicin (10 mcg)	< 12	13-14	> 15
Amikacin (30 mcg)	< 14	15-16	> 17
Ciprofloxacin (5 mcg)	< 15	16-20	> 21
Oflaxacin (5 mcg)	< 12	13-15	> 16
Cefotaxime (30 mcg)	Gm +ve < 14 Gm –ve < 10	15-22 11-15	> 23 > 16
Cefaperozone – Sulbactam (75mcg/30 mcg)	< 15	16-20	> 21
Imipenem 10 mcg	< 13	14-15	> 16
Vancomycin (30 mcg)			> 15

The diameters of the zones of inhibition were interpreted according to NCCLS standards for each organism.[28]

Identification of β-lactamase production [27]

The determination of β-lactamase production was done by iodometric method.

Procedure

Iodometric method

Requirements

i. 1% soluble starch solution prepared by dissolving the starch at 100°C.

ii. Iodine reagent consisting of 2.03g iodine and 5.32 g potassium iodide in 100 ml distilled water.

iii. Microtitre plate

Procedure

From an overnight incubation culture of the test organism, a heavy suspension was made (containing 10^9CFU/ml) in 100 mM sodium phosphate buffer at pH 7.3 containing penicillin 6g/litre, along with positive and negative controls.

Positive Control – *E.coli* ATCC 35218

Negative Control – *E.coli* ATCC 25922

The test and the control organisms were inoculated into the wells of a microtitre plate. After incubation for 1 hr at 37°C, two drops of freshly prepared 1% soluble starch solution was added to each well. A drop of Iodine reagent was then added. If blue colour was lost within 10 minutes, the presence of β-lactamase was inferred. If however, the blue persisted, the culture was considered to be β-lactamase negative.

DETECTION OF EXTENDED-SPECTRUM BETA-LACTAMASES (ESBL)

ESBLS are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (eg. ceftazidime, cefotaxime and ceftriaxone) and monobactams (eg. aztreonam) but do not affect cephamycins (eg. cefoxitin)

Cefpodoxime and ceftazidime have been proposed as indicators of ESBL production.

METHODS ADOPTED FOR DETECTION

Isolates showing a zone of inhibition < 27 mm for cefotaxime (30 mcg were tested for ESBL production as per NCCLS criteria, using standard control strains.[29]

1. COMBINED DISC METHOD [30]

Latest guidelines by NCCLS recommend a combined disc method as an indicator of ESBL production. For disc diffusion testing, $\geq$ 5mm increase in zone diameter of the antimicrobial agent tested in combination with betalactamase inhibitor (eg. clauvulanic acid, sulbactam) verses its zone when tested alone confirms an ESBL producing organism.

Combined Disc method using cefaperazone (75 mcg) and cefaperazone – sulbactum (75 mcg / 30 mcg) was performed for phenotypic confirmation of ESBL production.

2. DOUBLE DISC SYNERGY TEST [30]

In this test a lawn of the test strain on Mueller – Hinton agar was exposed to discs of ceftazidime (30 mcg) and augmentin (20 mcg amoxycillin / 10 mcg clavulanic acid) arranged in pairs. The discs were arranged so that the distance between them is approximately twice the radius of the inhibition zone produced by the cephalosporin tested on its own. After overnight incubation, the test strain was an ESBL producer if the inhibition zone around the cephalosporin disc was extended on the side nearest the augmentin disc.

Results

RESULTS

Table 1

Sex wise Distribution of patients with Haematological diseases

(n=94)

S. No.	Diseases	Male	Female	Total
1	AML	29	18	47
2	ALL	13	8	21
3	CML	2	2	4
4	NHL	4	2	6
5	CLL	1	-	1
6	H. Disorders	9	6	15
		58	36	94

Male preponderance was observed in patient with AML and ALL

No Significant difference was observed In Hematological Disorders.

Table 2

Age and Disease wise Distribution of patients with

Haematological diseases

(n=94)

Age	AML		ALL		NHL		CML		H. Disorders		CLL		Total
Group	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
10-20	5	3	6	5	-	1	-	-	1	-	-	-	21
21-30	9	5	3	2	3	-	2	1	4	3	-	-	32
31-40	5	4	2	1	-	1	-	1	1	1	-	-	16
41-50	6	3	1	0	1	-	-	-	1	-	-	-	12
51-60	2	2	-	-	-	-	-	-	2	1	1	-	8
61-70	2	1	1	-	-	-	-	-	-	1	-	-	5
Total	29	18	13	8	4	2	2	2	9	6	1	-	94

More then 50% of patients were below 30 years of age

Maximum Incidence in the age group of 21 – 30

Table 3

Episodes of infections in patients with Haematological diseases

(n=94)

Episodes	No. of Patients	Total episodes
1	78	78
2	12	24
3	3	9
4	1	4
Total	**94**	**115**

This works to 1.22 episodes of infection per patient per year.

Table 4

Comparative Analysis of Absolute Neutrophil count (ANC) vs Number of episodes vs Number of Isolation

(n=115)

ANC	Number of Episodes	Isolates Positive	%
>500 m^3	30	18	60 %
500 – 1000	12	4	33 %
1000 – 2000	14	2	14 %
>2000	59	9	15 %
Total	115	33	28.6 %

Isolation rate was found to be higher in patients with ANC less than 500/mm^3 of blood.

Table 5

Cullture positivity in various Samples during febrile episodes in the study group

(n=115)

Sample	Number tested	Bacterial isolates	Fungal isolates
Blood	115	19	-
Sputum	12	5	2
Oral ulcer	5	2	-
Wound	5	3	-
Tongue scraping	1	-	1
Faeces	1	1	-
Pleural fluid	1	-	-
Sputum AFB	12	-	-

Maximum number of isolates were from blood.

Table 6

Isolation of pathogens in patients with various

Haematological diseases

(n=94)

Diseases	No of Patient	Bacterial	Fungal	Total
AML	47	14	2	16
ALL	21	7	-	7
CML	4	2		2
H - Disorders	15	4	1	5
NHL	6	2	-	2
CLL	1	1	-	1
	94	30	3	33

Maximum number of isolates were from patients with AML

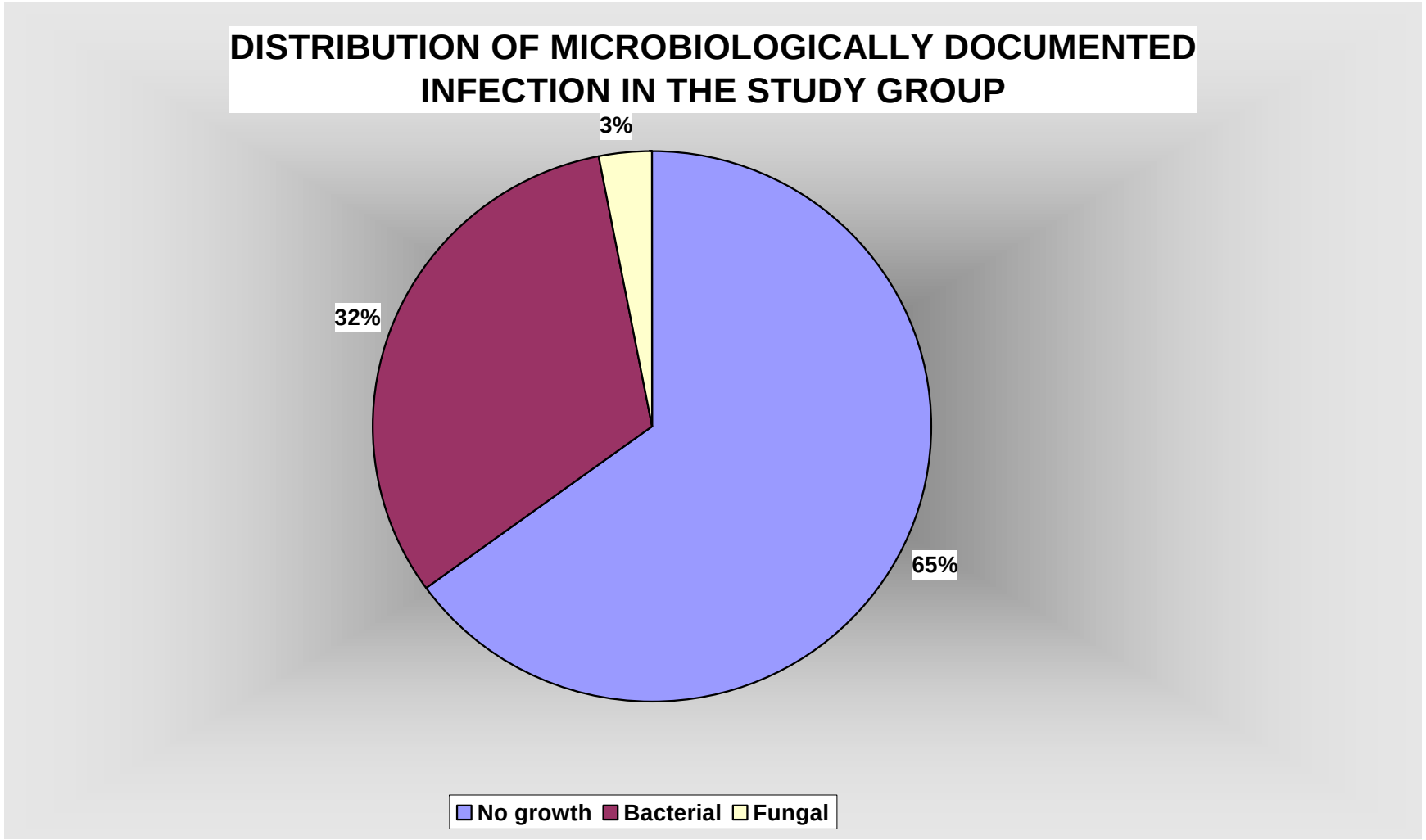

DISTRIBUTION OF MICROBIOLOGICALLY DOCUMENTED INFECTION IN THE STUDY GROUP
3%
32%
65%
No growth
Bacterial
Fungal

Table 7

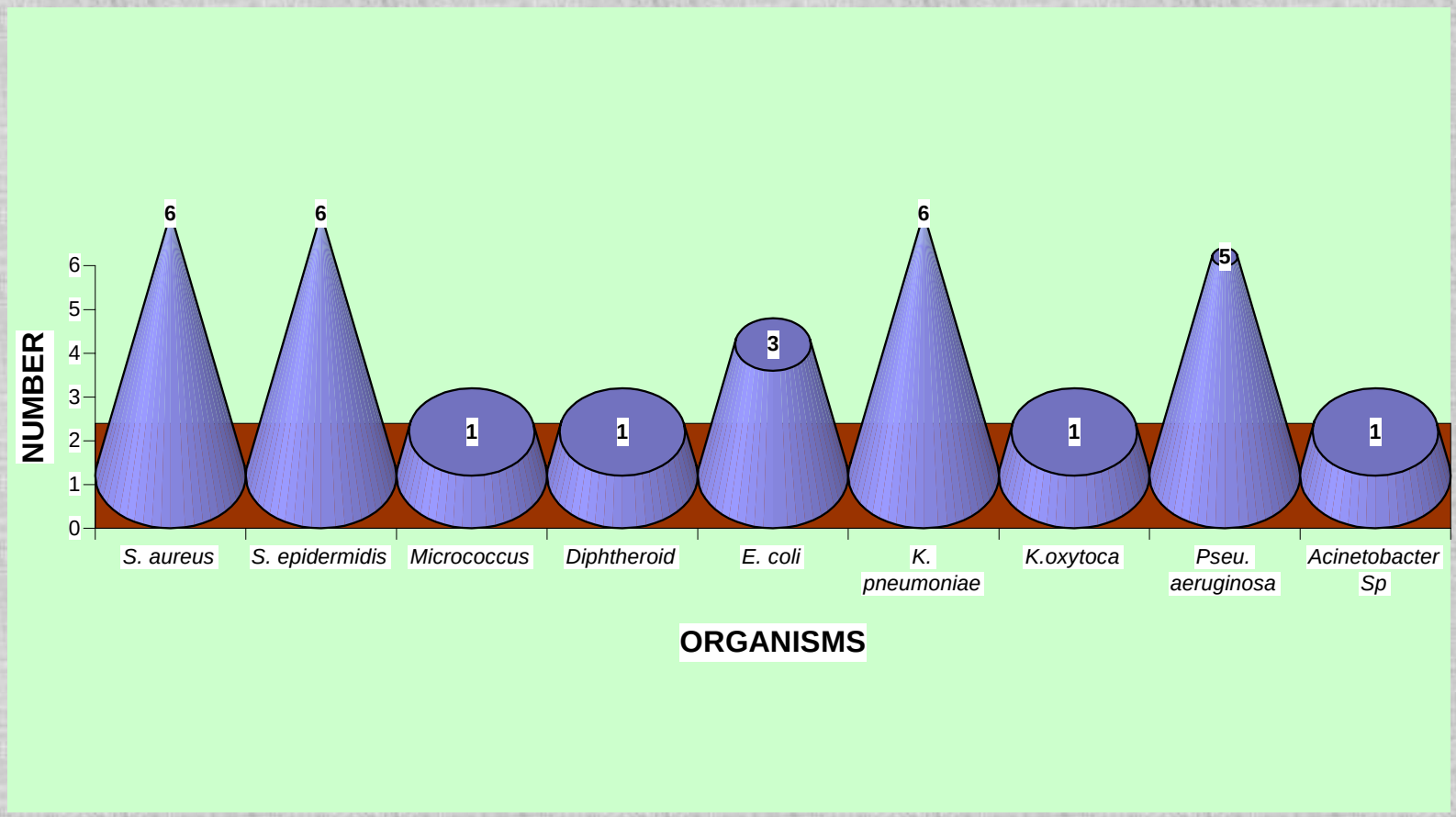
Blood culture Isolates from patients with Haematological diseases

(n=19)

Isolates	Number
S. aureus	5
S. epidermidis	6
Micrococcus	1
Diphtheroid	1
E. coli	2
K. pneumoniae	1
Pseu. aeruginosa	2
Acinetobacter Sp	1
Total	19

Predominant isolates from blood were *S. epidermidis* (31.6%) and *Staphylococcus aureus* (26.3%).

BACTERIAL PROFILE OF CULTURE POSITIVE CASES IN THE STUDY GROUP



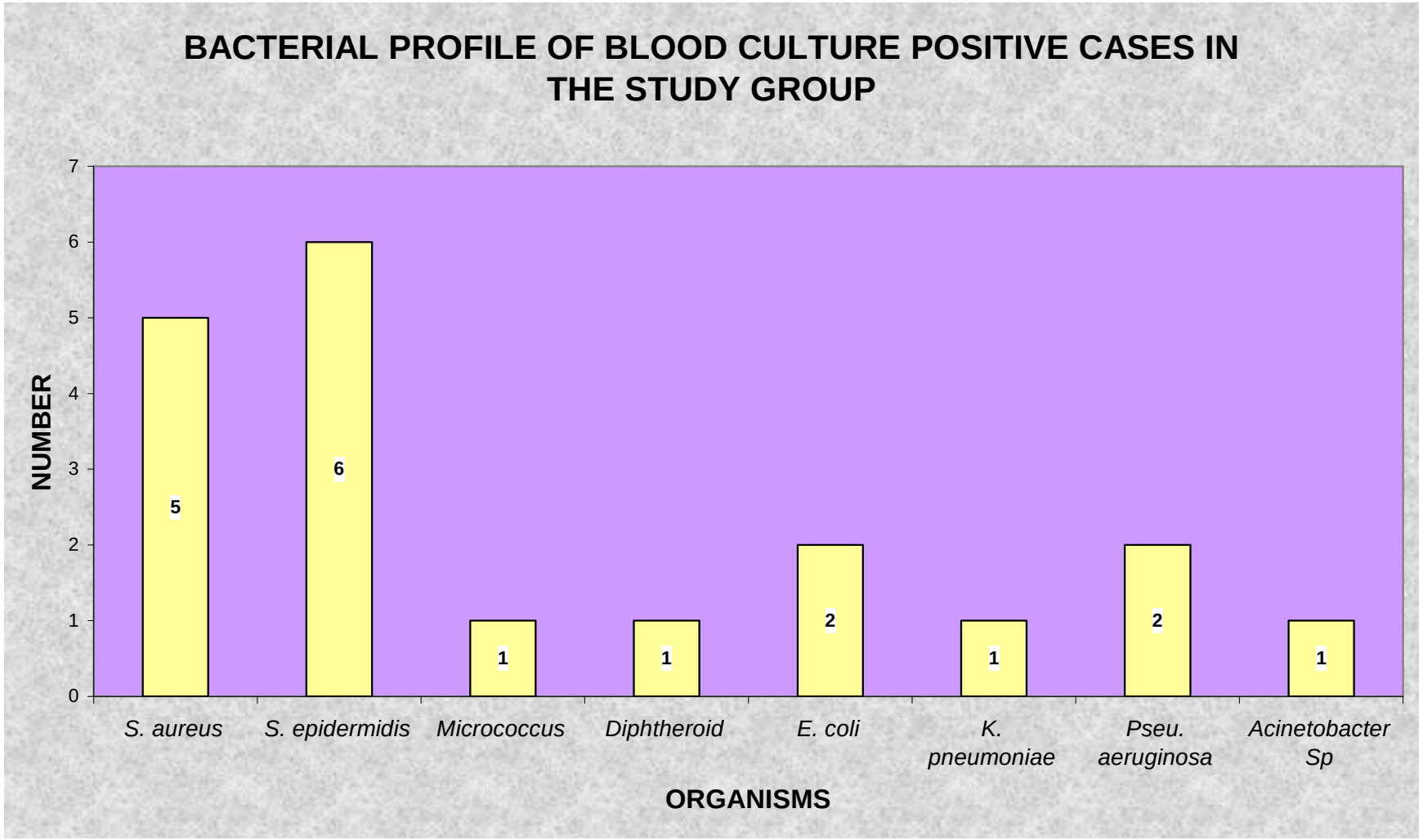

BACTERIAL PROFILE OF BLOOD CULTURE POSITIVE CASES IN THE STUDY GROUP
NUMBER
0
1
2
3
4
5
6
7
5
6
1
1
2
1
2
1
S. aureus
S. epidermidis
Micrococcus
Diphtheroid
E. coli
K. pneumoniae
Pseu. aeruginosa
Acinetobacter Sp
ORGANISMS

Table 8

Analysis of Organisms Isolated from Different

Specimens and Sites

(n=33)

Organism	Blood	Sputum	Oral ulcer	Skin wound	Tongue scraping	Faeces	Total
S. aureus	5	1	-	-	-	-	6
S. epidermidis	6	-	-	-	-	-	6
Micrococcus	1	-	-	-	-	-	1
Diphtheroid	1	-	-	-	-	-	1
E – coli	2	-	-	-	-	1	3
K. pneumoniae	1	2	1	2	-	-	6
K. oxytoca	-	1	-	-	-	-	1
Pseu. aeruginosa	2	1	1	1	-	-	5
Acinetobacter Sp	1	-	-	-	-	-	1
Apergillus flavus	-	2	-	-	-	-	2
Candida albicans	-	-	-	-	1	-	1
Total	19	7	2	3	1	1	33

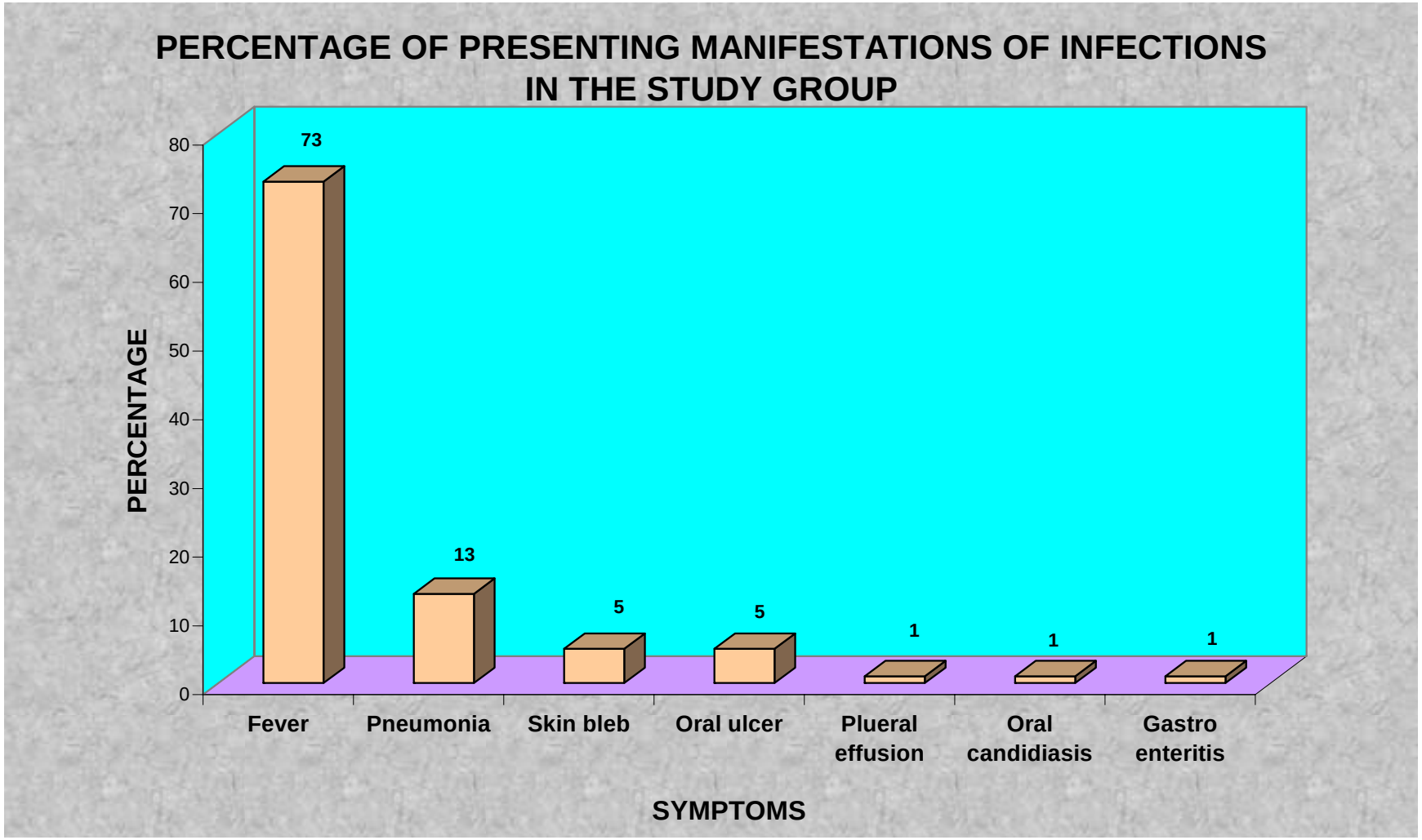

PERCENTAGE OF PRESENTING MANIFESTATIONS OF INFECTIONS IN THE STUDY GROUP
PERCENTAGE
80
70
60
50
40
30
20
10
0
73
13
5
5
1
1
1
Fever
Pneumonia
Skin bleb
Oral ulcer
Plueral effusion
Oral candidiasis
Gastro enteritis
SYMPTOMS

Table 9

Antibiotic-Susceptibility Pattern of Isolates from Study Group

(n=30)

Organism		Penicillin	Vanco-mycin	Amikacin	Genta-micin	ciprofloxacin	Ofloxacin	Cefotaxime	Cefaperozone -sulbactam	Imipenem	Piperacillin Tazobactum
S. aureus	6	-	6	6	-	3 (50%)	6		5 (83%)		
S. epidermidis	6	-	6	6		3 (50%)	46 (66%)	1 (16.6%)	5 (83%)		
Micrococcus	1	3 (50%)	1	1	-	1	1	1	1		
Diphtheroid	1	-	1	1	-	1	-	-	1		
E. coli	3	-		3	3	2 (66%)	3 (50%)	-	2	3	3
K. pneumoniae	6			4 (66%)	3	3	2	2 (33.3%)	6	6	6
K.Oxytoca	1			1	-	1	1	2	1	1	1
Pseu. aeruginosa	5			5	4	3 (60%)	1	2	5	5	5
Acinetobacter Sp	1			1	1	1	1	1	1	1	1

Amikacin was found to be the most useful antibiotic with 93% effectiveness.

Table 10

Analysis of Infective causes for Mortality in the Study group

(n=94)

Total Number of Patient	Number of patients died	Death due to Infective Cause	Gram negative Bacilliin blood	Gram positive organism in Blood	Gram negative organism in other site	Systemic Fungal Infection	No organism Isolated
94	8	7	3	1	2	1	1

Table 11

Percentage of β-Lactamase production in Gram positive isolates from patients with Haematological diseases

(n=12)

Organism	Number of β-lactamase Producers	Percentage
S. aureus (6)	3	50
S. epidermidis (6)	4	66.6

Table 12

Percentage of ESBL Productions in Gram negative isolates from patients with Haematological diseases

(n=14)

Organism	Number of ESBL Producers	Percentage
E-coli (3)	1	33
Klebsiella pneumoniae (6)	3	50
Pseudomonas aeruginosa (5)	1	20

Table 13

Antibiotic susceptibility pattern of Multidrug Resistant Isolates in patients with Haematological diseases

(n=4)

Site of Isolation	Organism	Number isolated	Résistance pattern	Sensitivity pattern
Blood	*K. pneumoniae*	1	AK, Cip, OF GM, CEF	Cef – Sul, Imi
Sputum	*K. pneumoniae*	1	AK, Cip, GM, OF , CEF	Cef – Sul, Imi
	Psendomonas aeruginosa	1	GM, OF, Cip, CEF	AK, Cef – Sul, Imi
Pus	*K. pneumoniae*	1	GM, OF, Cip, CEF	AK, Cef – Sul, Imi

All the 4 isolates were found to be ESBL producers

AK - Amikacin

Cip - Ciprofloxacin

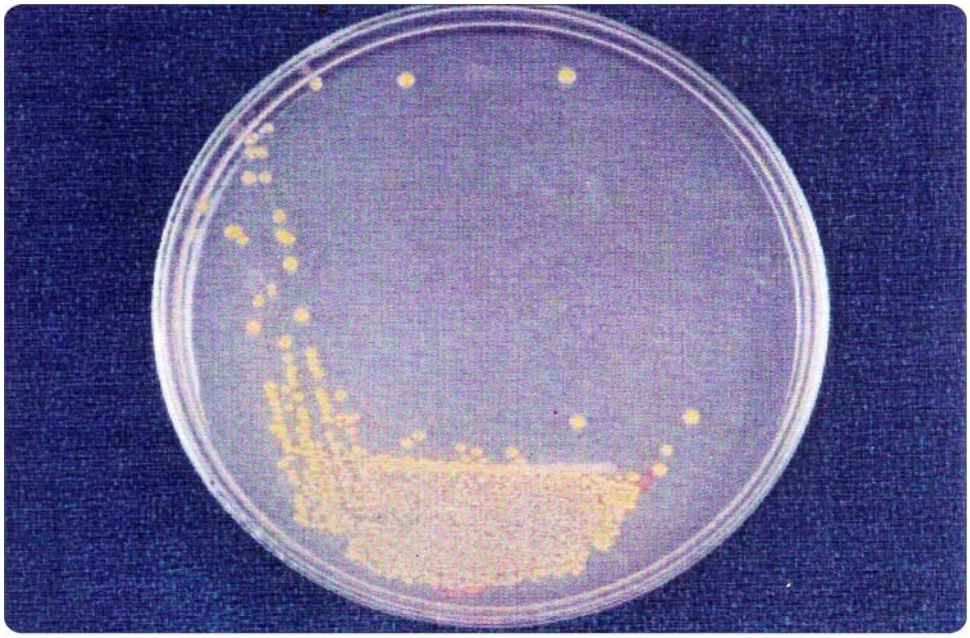
OF - Ofloxacin

GM - Gentamicin

CEF - Cefotaxime

Cef - Sul - Cefaperazone – sulbactam

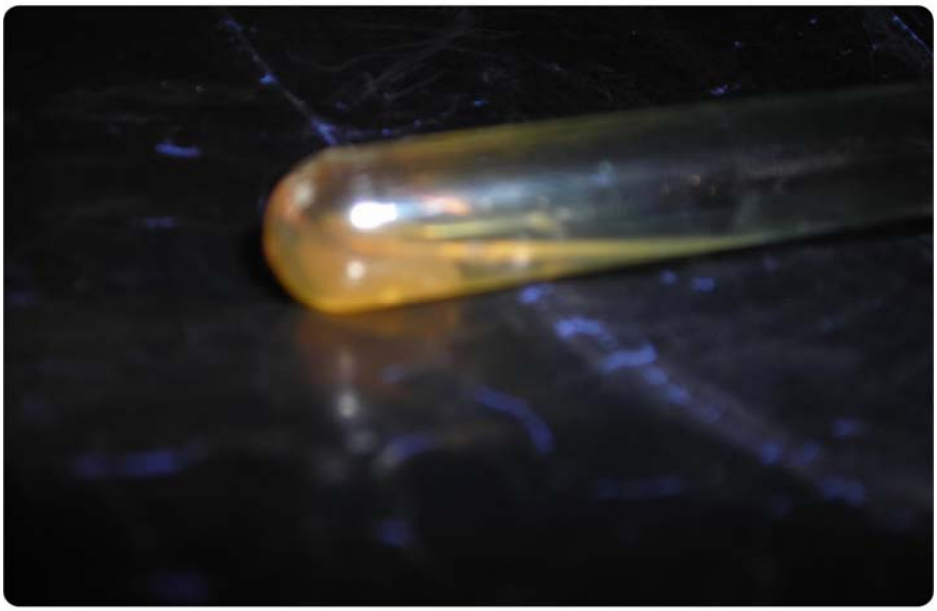
Imi - Imipenem



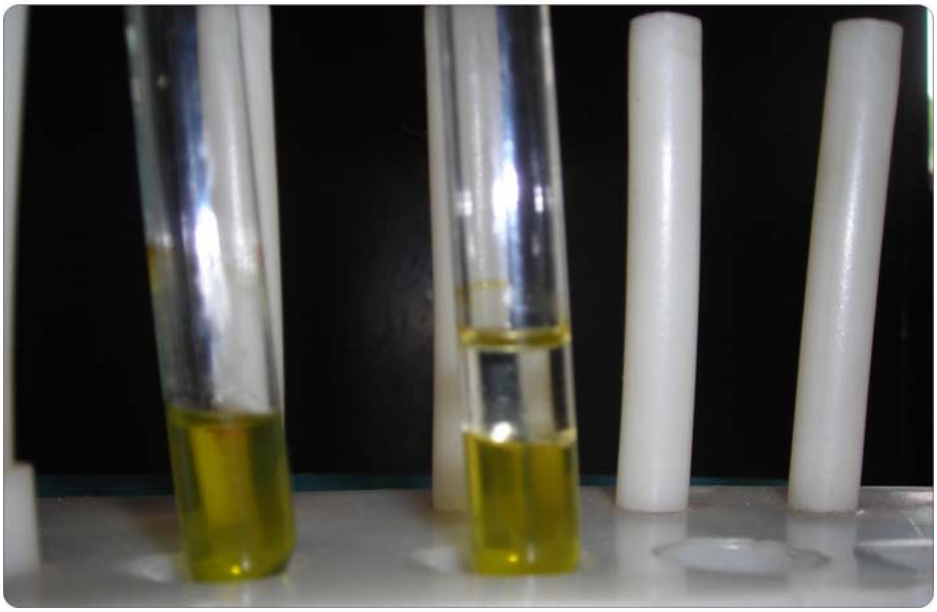
***Staphylococcus aureus* - NA Plate**

***Coagulase Negative Staphylococcus* - NA Plate**

Staphylococcus aureus
Tube coagulase test



O/F Test

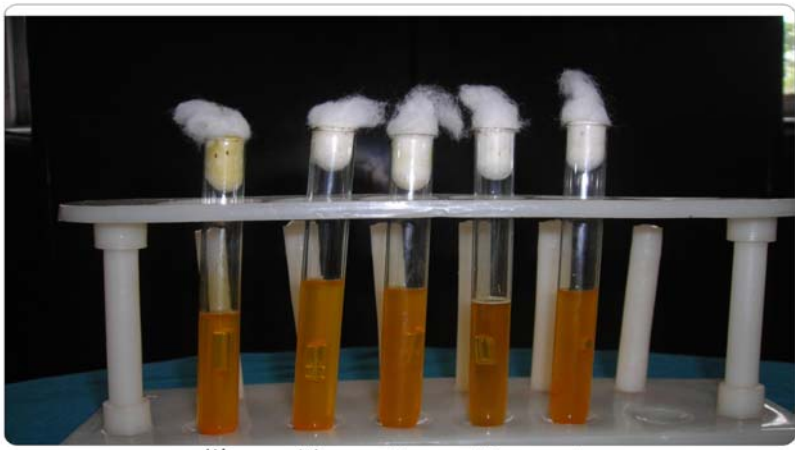


Klebsiella pneumoniae

MacConkey Agar Plate

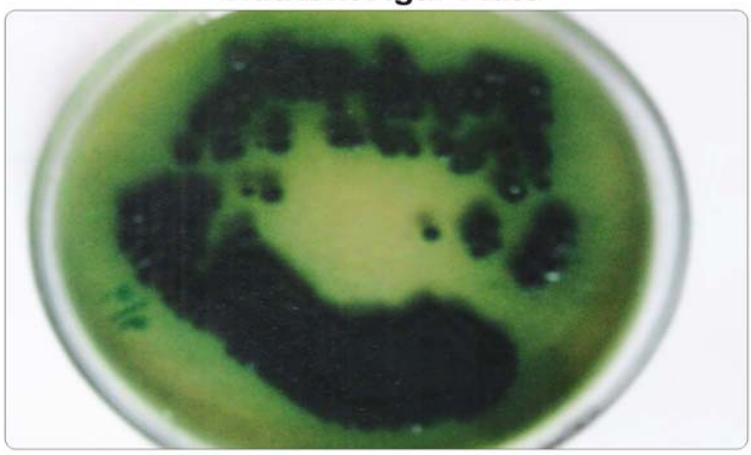
Biochemical Reactions

INDOLE MR VP CITRATE UREASE TSI



GLUCOSE LACTOSE SUCROSE MALTOSE MANNITOL

Pseudomonas aeruginosa
Nutrient Agar Plate

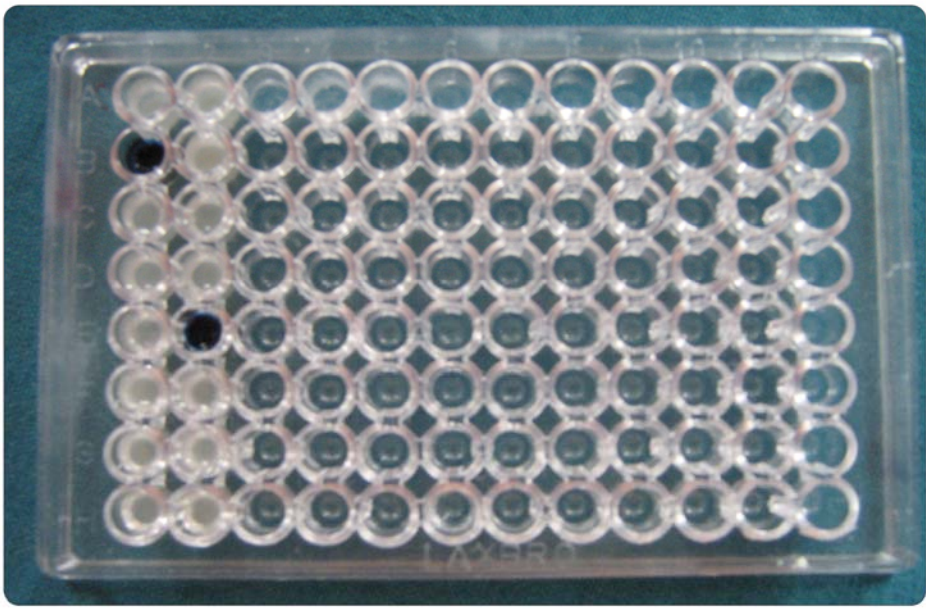


Biochemical Reactions

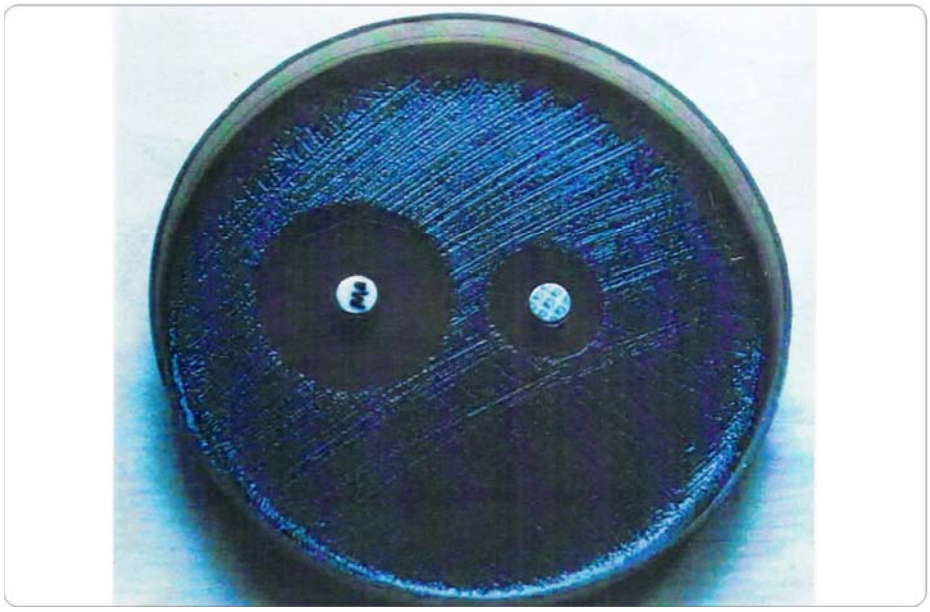
INDOLE TSI CITRATE UREASE MANNITOL MOTILITY

GLUCOSE LACTOSE SUCROSE MALTOSE MANNITOL

Beta-Lactamase Production
Iodometric Method



ESBL Production
Combined disc method



Discussion

DISCUSSION

The study undertaken by the Institute of Microbiology, Madras Medical College, Chennai, among patients admitted in the Department of Haematology, Government General Hospital, Chennai with haematological malignancies and disorders showed the following results.

This study was undertaken in 94 patients with a total of 115 episodes of febrile neutropenia.

Cases were distributed between the age group 10-70 years. Majority of patients were < 30 yrs of age with a maximal incidence between 21-30 yrs. This coincides with the accepted age group as described by Robbins, *et al.*[10]

50% of patients with acute lymphatic leukemia fell in the 10-20 years of age group. According to Robbins *et al* ALL is frequently diagnosed in patients <15years of age. [10]

There was a male preponderance constituting nearly three fifth of the total study population. Davidson states that males are affected more frequently than females, the ratio being about 3:2 in Acute Leukemia 2:1 in chronic lymphocytic leukemia and 1.3:1 in chronic myeloid leukemia.[11]

78 Patients suffered from a single episode of fever during the course of study, 12 patients suffered two such episodes and 3 patients had three episodes and one patient had four episodes of fever.

This works out to 1.22 episodes of infection per patient per year.

Robert B. Jerknes *et al* quotes an overall incidence of 0.25 – 0.50 episodes per patient per year increasing up to 1.8 episodes per year in patients who have advanced disease.[31]

Fever was the main presenting manifestation in 73% of patients in this study. This correlates well with the study by Khan M.A *et al* (2002) who reported, that 86% of patients presented with fever in the similar study group.[32]

Other presenting manifestations were, pneumonia in 13% of patients, skin bleb and oral ulcer each in 5% of patients, gastroenteritis, pleural effusion and oral candidiasis each in 1% of patients. These findings were similar to the study by Jagarlamudi R. *et al* (1998) who reported that respiratory infections were the most common infections next to fever followed by skin, soft tissue, GIT and genitourinary tract infections in order of decreasing frequency. [33]

In the present study microbiologically documented infection rate was 35% of which 32% was bacterial and 3% was fungal in etiology.

This correlates well with the study of A.K. Bilolikar *et al* (2000), where Microbiologically Documented Infection rate was 32%. [34]

Meunier *et al* had reported an incidence of 25–30% microbiologically documented infection in neutropenic patients.[35]

Das P.K. *et al* had reported that in his study Bacterial infection predominated followed by fungal and parasitic infection.[36]

In this study bacteraemia was observed in 16.5% of febrile episodes. Kumar L *et al* (1998) reported 13.72% of septicemic rate in his study at department of medical oncology ALLMS, New Delhi. [37]

Khan M.A. *et al*, (2003) reported septicemia in 16.1% of patients in his study.[32]

Gram positive pathogens are the predominant cause of infection in Neutropenic patients.[38]

In this study, Bacteraemia due to gram positive organisms accounts to 68.4% and due to gram negative organism accounts for 31.6%. This correlates well with the study of Jagarlamudi R. *et al*, 2006.[33]

Staphylococcus epidermidis was isolated in 31.6% and Staphylococcus aureus was isolated in 26.3% of all bacteraemias.

This Correlates with the increasing incidence of CONS bacteraemia in many centres.[19,39]

The most prevalent species of CONS was *S. epidermidis*. [40]

The spectrum of gram negative isolates includes, *E.coli* (2) *Pseudomonas aeruginosa* (2), *K.pneumoniae* (1), *Acinetobacter Sp*. (1). This correlates well with the study of Cordonnier C. *et al*, (2005) [41] and Kumar. L. *et al*, (2002)[37]

Patient with gram negative bacteraemia have a poor prognosis and higher mortality [42,43]. Hence all regimens are chosen to combat gram negative sepsis.[44] This may explain the shift from gram negative bacteraemia to isolation of gram positive organisms.

Factors considered responsible for the shift towards gram positive isolates include.[4]

1. Aggressive chemotherapy (Where ever cytarabine is given)
2. Radiotherapy causing mucositis
3. Profound and prolonged neutropenia
4. Unrecognized herpetic infection of the mucous membrane
5. Increased use of long dwelling intravenous catheters

6. H_2 antagonists

7. Use of cotrimoxazole and newer quinolones in neutropenic patients (Giamorellau and Antoniadou Pg 460).

Patients included in this study received H2 antagonists along with chemotherapy.

Fungal isolates in this study were from sputum and from tongue scrapings. Two were *Aspergillus flavus* and one was *Candida albicans*. No fungaemia was observed.

This is similar to the findings of Pagano L *et al* (2006) that, most common pathogen associated with fungal pneumonia are *Aspergillus* and *Zygomycetes*.[45]

The study population were put on regimen consisting of Cefotaxime which was changed to Fluconazole, Piperacillin-Tazobactam and Amikacin if the patient remained febrile and showed signs of failure to respond to the first regimen.

This may be responsible for the absence of fungaemia in this study.

No anaerobic organisms were isolated in this study. This reflects the less percentage of anaerobes causing infection in these neutropenic patients. [43]

Sputum specimens from patients with clinical pneumonia were subjected to sputum culture. Out of the 12 sputum samples, 3 gram negative isolates and one gram positive isolate and 2 isolates of *Aspergillus flavus* were obtained. This workout to 58% of microbiologically documented infection in pneumonia. This finding is similar to Fernandez *et al* (1999) [46]. This also reflects the fact that interstitial pneumonia of unknown cause could develop in patients with various hematological malignancies especially at recovery phase of chemotherapy.[47]

None of the 12 sputum were positive for AFB by Ziehl Neelson staining method. This may have been due to the fact that most of the patient had received broad spectrum antibiotic (including Amikacin) as part of empirical antibiotic therapy, to which the AFB may have been sensitive.

From one patient, in the first febrile episode *Klebsiella pneumoniae* was isolated from the sputum, and in the second febrile episode *Aspergillus flavus* was isolated.

This reflects the role of prolonged neutropenia and Antibiotic prophylaxis as predisposing factors for systemic fungal infection by replacing the normal flora.[48]

Bacterial infections are a major cause of illness and death.[55]

Septic shock and death due to Blood Stream Infection occurred more frequently in the group of neutropenic patients.[50]

In this study out of 94 patients, 8 died with a mortality rate of 8.5%. This correlates with the finding of Jagarlamudi. R. *et al* (2006)[33,51]

In the present study the important risk factor for mortality was infection, in 7 out of 8 patients (87%) which correlated with the study by Robert Bjerknes in which Infection alone accounted for 70% of all deaths.[31]

Out of the 5 gram negative isolates associated with mortality 4 isolates were ESBL producer and in these 4 isolates 3 were multidrug resistant. Mortality due to multidrug resistant gram negative bacteraemias was higher in comparison to bacteraemias due to susceptible organism[53]. This correlates well with the findings of Krcmeryr. *et al* (1998)[52]

One patient died with systemic fungal infection. Invasive pulmonary aspergillosis is a major cause of morbidity and mortality in neutropenic patients.[50]

Since chemotherapy induced neutropenia is more severe in elderly patients, increasing age was associated with increased mortality from bacteremia in patients with haematological malignancies.[55]

In this study, 85% of all deaths occurred in the age group between 41-50 years. This correlates well with the study of Norgaard M. *et al*. (2006).[55]

In the present study, 67% of bacterial isolates and all the 3 fungal isolates were obtained from patients with ANC less than 1000/mm^3 of blood.

Absolute neutrophil counts have been found to be the most useful prognostic indicator for development of infection.

Giamarellou and Antoniadou regard neutrophil counts less than 100 / mm^3 as having high risk of infections and mortality.[49]

D.Hiwase, A.Mukhopadhya *et al* report an increased risk of infection at counts <1000 /mm^3 with life threatening infection at levels of 100 /mm^3 [35].

Since increased duration of neutropenia leads to risk of infection, it is a universal practice to start the patients on prophylactic empirical antibiotic therapy along with antifungals.

Antibiogram was done for the 30 bacterial isolates and susceptibility pattern noted.

Amikacin was found to be the most effective antibiotic with 93% sensitivity to all the isolates. This is similar to the finding of A.K.Bilolikar *et al* (2000) [34]

Cefaperazone-sulbactam was 90% sensitive except 2 isolates of gram positive cocci and one isolate of *E-coli.*

Only 37% of all isolates were sensitive to cefotaxime.

80% of isolates of *Staphylococcus aureus* and 50% of *S.epidermidis* isolates were found to be resistant to oxacillin. Out of this 50% of *S.aureus* and 66.6% of *S.epidermidis* were found to be β – Lactamase producers.

This is in accordance with the findings of Kirby JT, *et al* (2006). [58]

Three isolates of *Klebsiella pneumoniae* from blood, sputum and pus and one isolate of *Pseudomonas aeruginosa* from sputum were found to be multidrug resistant. All these four isolates and one isolate of *E.coli* from blood were found to be ESBL – producers.

All the gram positive organisms including Methicillin resistant isolates were sensitive to Vancomycin [56,57] This finding was found to be statistically significant with p value 0.003.

All the gram negative organism including ESBL producers were sensitive to Imipenem and Piperacillin-Tazobactam combination. This was found to be statistically significant with p value 0.01.

Out of 4 gram negative bacterial isolates from sputum 2 were found to be ESBL producers. This is similar to the study of S.Shawgi *et al* (2004) [59]

These organisms are resistant to all cephalosporins and exhibit varying and unpredictable degrees of sensitivity to aminoglycosides and quinolones. The carbapenems are active against these pathogenes [44]. This points out to the emergence of ESBL producing organisms as an important cause of infection in these patients. This also highlights the importance of using antibiotics that are capable of neutralising the ß–lactamase produced by these organisms and incorporating them into newer empirical antibiotic combinations.

Timely identification of the causative organisms aids in the successful management of these patients.

Summary

SUMMARY

- In the present study undertaken in patients with Haematological malignancies and disorders associated with neutropenia, more than 50% of patients were below 30 years of age.
- Male preponderance was observed in patients with AML and ALL
- Infection rate was found to be 35%.
- Bacterial infections were predominant constituting 32% followed by fungal infection 3%.
- Bacteraemia was observed in 16.5% of microbiologically documented infection.
- 68.4% of bacteraemias were due to Gram positive cocci and 31.6% of bacteraemias were due to Gram negative bacilli.
- *Staphylococcus epidermidis* were isolated in 31.6% and *Staphylococcus aureus* were isolated in 26.3% of all bacteraemias.
- 80% of *staphylococcus aureus* isolates and 50% of *S.epidermidis* Isolates were Methicillin resistant.

- All the Methicillin resistant isolates were sensitive to Vancomycin
- 31.25% of Gram negative isolates were found to be ESBL producers. All ESBL producers were sensitive to Imipenum, Piperacillin-Tazobactam
- Multidrug resistance was observed in 3 isolates of *Klebsiella sp* and one isolate of *Pseudomonas sp.*
- Amikacin was effective against most of the isolates (96%)
- Cefaperozone-sulbactam and Ofloxacin were found to be the other effective antibiotics
- There was no significant difference in the infection rate in patients with Haematological malignancies and disorders.
- Absolute neutrophil count < 500/mm^3 was found to be an important risk factor for infection.
- The overall mortality was 8.5%. Infection was the major risk factor for mortality in 87% of cases of death.

Conclusion

CONCLUSION

- Bacterial infections are the major cause of morbidity and mortality in patients with haematological malignancies during chemotherapy induced neutropenia, and in patients with Haematological disorders associated with neutropenia.

- In this study, the spectrum of isolates among febrile neutropenic patients, appears to be shifting towards Gram positive microorganisms.

- Therapy induced neutropenia is the most important risk factor for infectious complications in patients with haematological malignancy.

- Increasing age was found to be associated with increased mortality from bacteraemia.

- Mortality due to multidrugresistant Gram Negative bacteraemia was higher in comparison to bacteraemia due to susceptible organisms.

- Since bacterial infections were predominant cause of mortality, close monitoring of infection should be done by repeated blood cultures, and emergence of drug resistance prevented by doing periodical modification of empiric antibiotic regime.

Annexure

PROFORMA

Name:	In Patient No:
Age:	Address:
Sex:	Occupation:
	Diagnosis:

Presenting Complaints

Duration of illness

Previous History of similar illness or treatment

Personal History

Family History

Clinical Examination – General Examination

Systemic examination

Diagnosis

Treatment

Antibiotics

Chemotherapy

Radiotherapy

Bone marrow transplantation

Laboratory Investigations

1. Complete Hemogram

Total count, Differential count, Hemoglobin percentage,

Packed cell volume, Platelet count,

2. Biochemical tests

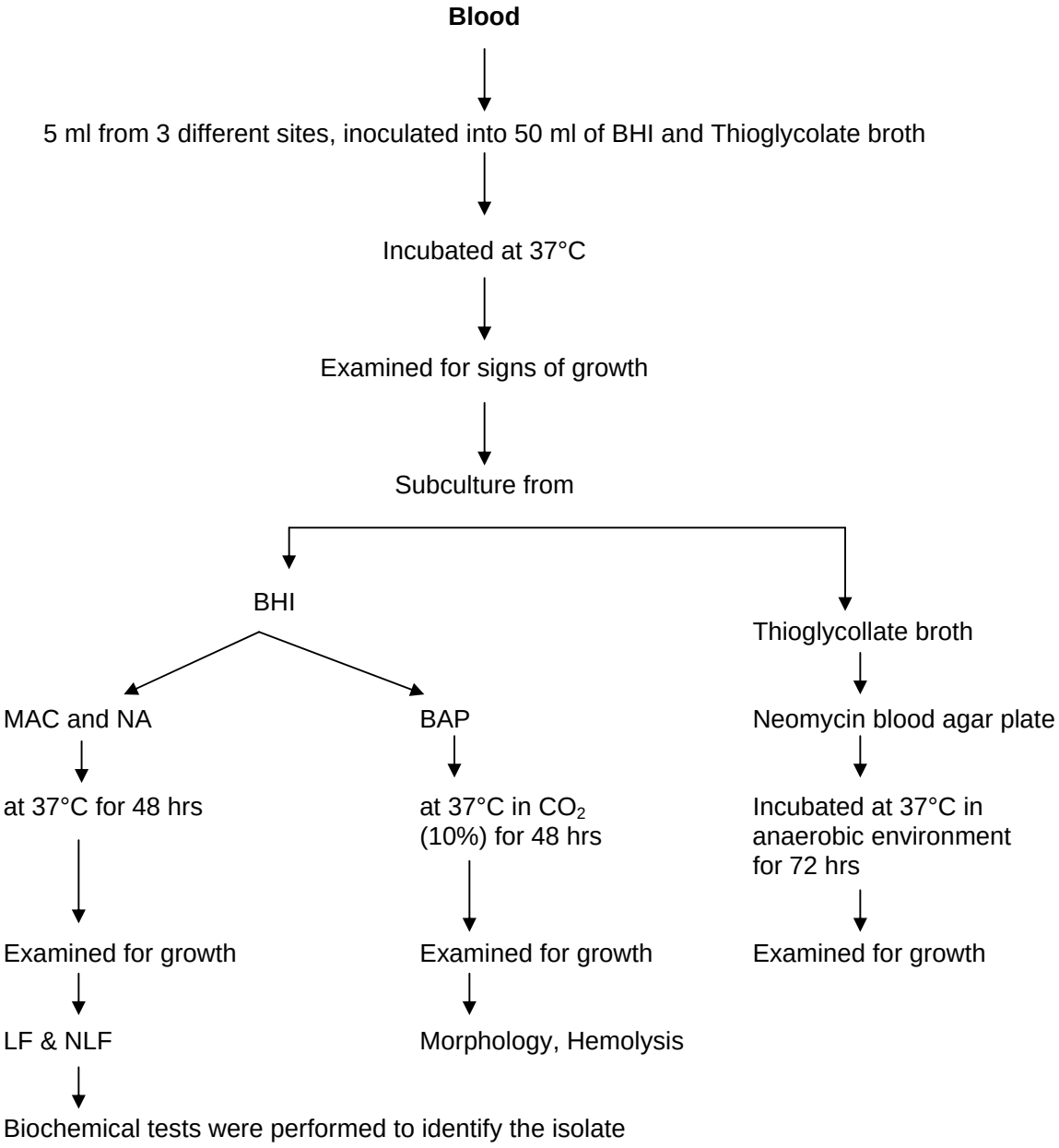
3. Microbiological Methods

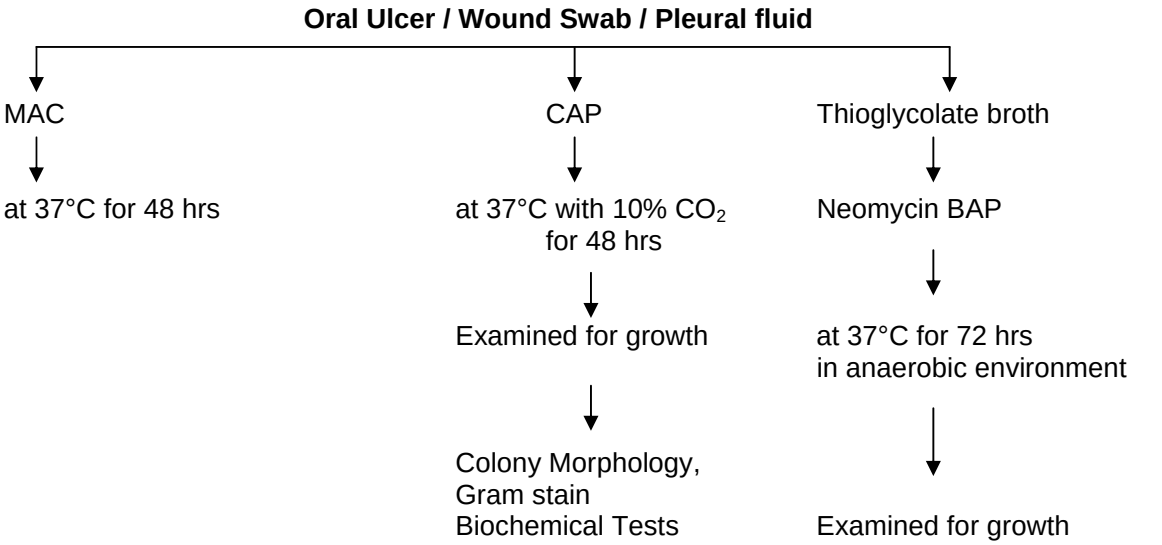
Microscopy – Gram stain

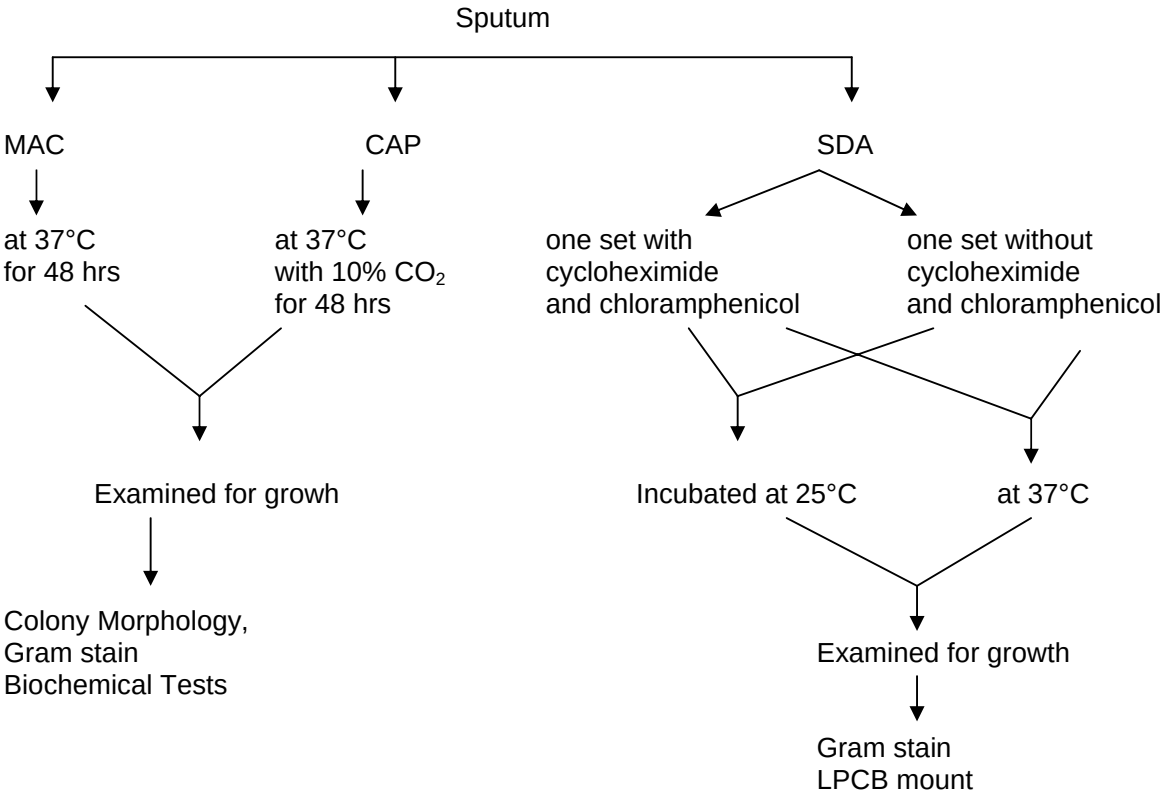
Culture

Antimicrobial Susceptibility test

PROCESSING OF SAMPLE




Oral Ulcer / Wound Swab / Pleural fluid
MAC
CAP
Thioglycolate broth
at 37°C for 48 hrs
at 37°C with 10% CO_2 for 48 hrs
Neomycin BAP
Examined for growth
at 37°C for 72 hrs in anaerobic environment
Colony Morphology, Gram stain Biochemical Tests
Examined for growth


Sputum
MAC
CAP
SDA
at 37°C for 48 hrs
at 37°C with 10% CO_2 for 48 hrs
one set with cycloheximide and chloramphenicol
one set without cycloheximide and chloramphenicol
Examined for growh
Incubated at 25°C
at 37°C
Colony Morphology, Gram stain Biochemical Tests
Examined for growth
Gram stain LPCB mount

Appendix

APPENDIX 1

Brain Heart Infusion Broth (BHI)

This is reconstituted from the dehydrated form according to the manufacturer's instructions

		gm / litre
Calf brain infusion	-	200.00
Beef infusion	-	250.00
Proteose peptone	-	10.00
Dextrose	-	2.00
Sodium chloride	-	5.00
Disodium phosphate	-	2.50

Final pH 7.4 ± 0.2

Preparation

37 gms were suspended in 1000 ml distilled water and dispensed into bottles. They were sterilized by autoclaving at 121°C (15 lbs pressure) for 15 min.

Thioglycolate Broth

Yeast extract	-	5 g
Casein hydrolysate	-	15 g
Glucose	-	5.5 g
L.cystine	-	0.5 g
Agar	-	0.75 g
Sodium chloride	-	2.5 g
Sodium thioglycollate	-	0.5 g
Resazurin sodium solution		
1 in 1000, freshly prepared	-	1 ml
Distilled water	-	1 litre

All the ingredients other than thioglycollate and resazurin were dissolved in distilled water by steaming at 100°C. Next thioglycollate was added, pH adjusted to 7.3. Then resazurin solution was added, mixed thoroughly, distributed in glass tubes and sterilized at 121°C for 15 min. Cool at once to 25°C and stored in the dark.

Selenite F Broth

Sodium hydrogen selenite	4 g
Peptone	5 g
Lactose	4 g
Disodium hydrogen phosphate	9.5 g
Sodium dihydrogen phosphate	0.5 g
Sterile water	1 litre

All the ingredients were dissolved in sterile water with sterile precautions. The yellowish solution in about 10 ml amounts distributed into screw-capped universal bottles. Steamed for 20 min at 100°C pH adjusted to 7.1.

APPENDIX 2

Elisa for HBs Ag (Microscreen Elisa test kit)

1. All the reagents of the kit were brought to room temperature except colour reagent.
2. 100 µL of sample diluents was added to all wells except blank well.
3. 100 µL of negative, positive controls and samples were added to the respective wells.
4. Incubated at room temperature (20-30°C) for 60 minutes.
5. The well contents were discarded and washed 5 times with 30 seconds soak time at each step.
6. 50 µL of conjugate stabilizer and 100 µL of conjugate was added to all wells except blank well and incubated at room temperature for 30 min.
7. It was washed 5 times.
8. 100 µL of colour reagent was added to all wells and incubated at room temperature in dark for 30 min.
9. 100 µL of stop solution was added to all wells
10. Results were read at 450 nm.

Test validity: Test was valid when

Negative control < 0.2

Positive control > 1.00

Reagent blank < 0.100 when read in bichromatic mode

(450-630 nm)

Cut off calculation

The cut off was calculated based on the mean absorbance of 3 negative controls and addition of a factor 0.10 ie.

Cutoff = $NC\bar{X}$ + 0.10

Interpretation of results

1. All samples with absorbance less than out on value were considered non reactive for HBs Ag.

2. Samples with absorbance more than cut off value were considered reactive

ELISA for HIV (Microlisa – HIV)

1. 100 µL of sample diluent was added to A1 well as blank

2. 100 µL of negative control was added to B-1 and C-1 wells

3. 100 µL of positive control was added to D-1, E-1 and F-1 wells

4. 100 µL of sample diluent was added in each well starting from G-1 followed by 10 µL of sample was added.

5. Applied cover seal and incubated at 37°C for 30 min.

6. The well contents were discarded and washed 5 times with 30 seconds soak time at each step, with working wash solution.

7. 100 µL of working conjugate solution was added to each well including A-1.

8. Applied cover seal, incubated at 37°C for 30 min

9. Well contents were aspirated and washed for 5 times

10. 100 µL of working substrate solution was added in each well including A-1

11. Incubated at room temperature for 30 min in dark (20-30°C)

12. 50 µL of stop solution was added to all wells.

13. Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well.

Cut off calculation

The cut off value is calculated by adding mean negative control ($NC\bar{x}$) and Mean Positive Control ($PC\bar{x}$) as and the sum is divided by six.

$$\text{Cut off value} = \frac{NC\bar{x} + PC\bar{x}}{6}$$

Interpretation of Results

Test specimens with absorbance value less than the cutoff value are non-reactive and considered as negative for anti-HIV

Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV

APPENDIX 3

Widal Agglutination test

Doubling dilution of serum was done for O,H,AH and BH from 1:25, upto 1:400

Antigen controls were also putup

↓

Equal quantity of corresponding antigen was added

↓

Incubated overnight at 37°C

↓

Results were read for ‘H’ and ‘O’ agglutinin

The antigens used S.typhi O, S.typhi H, S.paratyphi AH S.paratyphi BH were obtained from King Institute of Preventive medicine (KIPM), Guindy.

Interpretation of results

‘H’ agglutination seen as formation of loose, cotton wooly clumps

‘O’ agglutination seen as granular deposit at the bottom of the tube

Antigen controls with no agglutination seen as compact button at the bottom of the tube.

The criteria for diagnosis were,

'O' agglutination titre of ≥ 1 in 100

'H' agglutination titre of ≥ 1 in 200

MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT) FOR LEPTOSPIROSIS

MSAT was performed using pooled suspension of leptospiral killed antigens. The following leptospiral serogroups were used Icterohaemorrhagiae, Autumnalis, Australis, Hebdomadis, Pomona, Patoc, Lousiana, Grippotyphosa.

Procedure

8 µL of phosphate buffered saline and 5 µL of patients serum was placed on a depression slide and 12 µL of heat killed pooled antigen was added.

The slide was rotated at 180 rpm for 4 min on a rotator. It was examined macroscopically for the presence of agglutination and confirmed by DFM. Positive and negative controls was also put up.

The results of MSAT were read as follows:

Negative	:	No agglutination and uniformity of serum antigen mixture
1+	:	25% agglutination
2+	:	50% agglutination
3+	:	Obvious agglutination but partial clearing of suspension
4+	:	Clumps of agglutination with complete clearing of leptospiral antigen suspension. An agglutination of $\geq$ 2+ is considered as positive.

STANDARD AGGLUTINATION TEST (SAT)

FOR BRUCELLOSIS

Two-fold dilutions of serum was made from 1 in 20 to 1 in 640 in 0.4% phenol saline for Br.melitensis and Br.abortus. Antigen controls were also putup.

↓

Equal quantity of standardized antigen suspension was added

↓

Incubated at 37°C

↓

Results were read after 48 hrs

The antigens were obtained from king institute of preventive medicine Guindy.

Interpretation

Reactions of partial or complete clearing with agglutination visible by the naked eye should be reported as positive.

Controls with no agglutination seen as compact button at the bottom of the tube.

A titre of 160 or more is considered positive.

APPENDIX 4

Leishman's stain

Ingredients

0.15 gm of tablet

Dissolve in 100 ml of absolute methyl alcohol

APPENDIX – 5

Blood Agar

Sterile defibrinated sheep blood	- 10 ml
Nutrient Agar (melted)	- 100 ml

Sterile Nutrient agar was melted cooled to about 45-50°C, 7-10ml of sterile defibrinated sheep blood was added. Mixed well and 15 ml of blood agar was poured in petri distress.

MacConkey Agar

Peptone	-	20 g
Sodium taurocholate	-	5 g
Water	-	1 litre
Agar	-	20 g

Neutral red solution

2% in 50% ethanol	-	3.5 ml
lactose, 10% aqueous solution	-	100 ml

All the ingredients except lactose and neutral red were dissolved in distilled water by heating. pH adjusted to 7.5. Lactose and neutral red were added and sterilized by autoclaving at 121°C for 15 minutes.

Nutrient Agar

		Gms / litre
Peptic digest of animal tissue	-	5.00
Beef extract	-	1.50
Yeast extract	-	1.50
Sodium chloride	-	5.00
Agar	-	15.00

28.0 grams were suspended in 1000 ml of distilled water. Boiled to dissolve the medium completely. Final pH adjusted to 7.4 ± 0.2 sterilized by autoclaving at 121°C for 15 minutes.

Neomycin Blood agar (NBA)

Nutrient Agar	-	100 ml
Blood	-	7 ml
Neomycin 1% solution	-	0.25 ml

Sterlized nutrient agar, cooled to 45-50°C, then sterile blood and neomycin solutions were added. Mixed thoroughly and poured 25 ml per plate.

Chocolate Agar Plate

Sterile Refibrinated blood	-	10 ml
Nutrient Agar (Melted)	-	100 ml

When the temperature was about 75°C, sterile blood was added with constant agitation, in the water bath. Heating was continued till the blood becomes chocolate brown in colour. Cooled to about 50°C and poured about 15 ml into petri dishes.

Xylose lysine deoxycholate (XLD) agar

Yeast extract	3.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
L-lysine Hcl	5.0 g
Sodium Chloride	5.0 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Phenol red	0.08 g

Agar	15 g
Water	1 litre

All the ingredients except deoxycholate, thiosulphate and ferric ammonium citrate were dissolved in the water by autoclaving. Cooled to 50°C and 20 ml of a solution of sodium thiosulphate 34% and ferric ammonium citrate 4% and 25ml of a solution of sodium deoxycholate 10% were added, mixed. pH adjusted to 6.9 and at once poured in plates. Complete medium in powdered form is also available commercially.

Mueller Hinton Agar

Beef Infusion	-	300 ml
Casein hydrolysate	-	17.5 g
Starch	-	1.5 g
Agar	-	10 g
Distilled water	-	1 litre

The starch was emulsified in a small amount of cold water and poured into the beef infusion and the casein hydrolysate and the agar were added. The volume was made upto 1 litre with distilled water. The constituents were dissolved by heating gently at 100°C with agitation. The pH was adjusted to 7.4. Sterilized by autoclaving at 121°C and poured in plates.

APPENDIX 6

Media for Biochemical Identification of Bacteria

Oxidase	Catalase
Indole	Triple Sugar Iron agar
Citrate	Urease
Methyl red – Voges Proskauer	Nitrate reduction.
Phenyl alanine deaminase test,	Carbohydrate Fermentation media

1. Oxidase Reagent

Tetra methyl P- phenylene diamine dihydro chloride -1% aqueous solution.

2. Catalase Test

3% Hydrogen peroxide

3. Indole Reagent

Kovac's reagent

Para dimethyl amino benzaldehyde -10gm

Iso amyl alcohol -150ml

Hydrochloric acid -80ml

4. Triple Sugar Iron Agar

Sodium chloride 0.5gm

Yeast extract 0.5gm

Peptone 2gm

Agar 1.5gm

Distilled water 100ml

Distilled by keeping in boiling water bath and the following ingredients were added.

Lactose 1.0gm

Sucrose 1.0gm

Dextrose 0.1gm

Sodium thio suiphate 0.03gm

Ferrous suiphate 0.02gm

pH adjusted to 7.6. Phenol red 0.0024gm (2.4 ml of 1% solution) was added and distributed into test tubes in 4ml quantities and autoclaved. The tubes were kept in a slanting position so that to get a deep but and a short slant.

5. Simmon's Citrate Agar

Sodium chloride 5.0g

Magnesium sulphate 0.2g

Ammonium dihydrogen phosphate 1.0g

Potassium dihydrogen phosphate 1.0g

Sodium citrate 5.0g

Agar 20.0g

Bromothymol blue (1/500 aqueous solution) 40ml

Distilled water 100ml

The ingredients were mixed and pH adjusted to 6.9 sterilized by autoclaving and poured into tubes as slopes.

6. Christensen's Urea Agar

Urea solution

Sodium chloride -5.0g

Dextrose -1.0gm

Trypticase -1.0gm

Mono potassium phosphate -2.0gm

Urea -20.0gm

Distilled water -100ml

Phonol red 1% solution -1.2ml (in alcohol)

Urea agar base

Agar -1.5gm

Distilled water -90ml

The ingredients were dissolved in distilled water. pH adjusted to 6.8. Phenol red solution was added & sterilized by filtration. This is stock solution.

Agar was dissolved in distilled water and sterilized by autoclaving. Cooled to 45°C and 10ml of urea solution was added, dispensed in 3-5ml quantities and allowed to form a small butt and long slant.

7. Glucose Phosphate Broth (MR-VP medium)

Dipotassium phosphate 5.0gm

Glucose 5.0 gm

Distilled water 100ml

The above ingredients were suspended in distilled water and heated slightly to dissolve them. Sterilized at 115° C for 15 minutes.

8. Potassium Nitrate Broth

Potassium nitrate (KNO_3) 0.2gm

Peptone 5.0gm

Distilled water 100ml

The above ingredients were mixed and transferred into tubes in 5ml amount and autoclaved.

9. Phenyl Alanine Deaminase Test

Yeast extract	5g
Disodium hydrogen phosphate	1g
Agar	12g
Di-Phenylalanine	2g
Sodium chloride	5g
Distilled water	1 litre

pH adjusted to 7.4, distributed in tubes and sterilized by autoclaving at 121°C for 15 minutes, allowed to solidify as long slopes.

APPENDIX 7

Sabouraud Dextrose Agar (SDA)

Peptone	1 gm
Dextrose	4 gm
Agar	2 gm

Chloramphenicol 5 mg

Cycloheximide 50 mg

Distilled water 100 ml

All the ingredients were dissolved by boiling in a water bath. Cycloheximide was dissolved in 10 ml of acetone and added to the boiling medium. Similarly, chloramphenicol was dissolved in 10 ml of 95% alcohol and added to the boiling medium. The medium was removed from heating, mixed well and then dispensed in tubes and autoclaved at 121°C for 15 minutes. The pH adjusted to 5.4. The tubes were cooled in slanted position and stored in the refrigerator.

Abbreviations

ABBREVIATIONS

AFB	-	Acid Fast Bacilli
ALL	-	Acute Lymphatic Leukemia
AML	-	Acute Myleoid Leukemia
ANC	-	Absolute Neutrophil Count
BAP	-	Blood Agar Plate
BHI	-	Brain Heart Infusion
BSI	-	Blood Stream Infection
CDI	-	Clinically Documented Infection
CLL	-	Chronic Lymphocytic Leukemia
CML	-	Chronic Myleoid Leukemia
CONS	-	Coagulase negative staphylococcus
ESBL	-	Extended Spectrum ß-Lactamase
FUO	-	Fever of Unknown Origin
HBsAG	-	Hepatitis B Surface Antigen
HD	-	Haematological Disorders
HIV	-	Human Immunodeficiency Virus

HL	-	Hodgkin’s Lymphoma
LPCB	-	Lactophenol Cotton Blue
MAC	-	MacConkey Agar
NA	-	Nutrient Agar
NHL	-	Non-Hodgkin’s Lymphoma
NCCLS	-	National Committee for Clinical Laboratory Standards
SDA	-	Sabouraud Dextrose Agar
XLD	-	Xylose Lysine Decarboxylase

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